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Life in the Cold: Proteomics of the Antarctic Bacterium *Pseudoalteromonas haloplanktis*

Florence Piette, Caroline Struvay,
Amandine Godin, Alexandre Cipolla and Georges Feller
*Laboratory of Biochemistry, Center for Protein Engineering, University of Liège
Belgium*

1. Introduction

It is frequently overlooked that the majority (>80%) of the Earth's biosphere is cold and permanently exposed to temperatures below 5 °C (Rodrigues & Tiedje, 2008). Such low mean temperatures mainly arise from the fact that ~70% of the Earth's surface is covered by oceans that have a constant temperature of 4°C below 1000 m depth, irrespective of the latitude. The polar regions account for another 15%, to which the glacier and alpine regions must be added, as well as the permafrost representing more than 20% of terrestrial soils. All these low temperature biotopes have been successfully colonized by cold-adapted microorganisms, termed psychrophiles (Margesin *et al.*, 2008). These organisms do not merely endure such low and extremely inhospitable conditions but are irreversibly adapted to these environments as most psychrophiles are unable to grow at mild (or mesophilic) temperatures. Extreme psychrophiles have been traditionally sampled from Antarctic and Arctic sites, assuming that low temperatures persisting over a geological time-scale have promoted deep and efficient adaptations to freezing conditions. In addition to ice caps and sea ice, polar regions also possess unusual microbiotopes such as porous rocks in Antarctic dry valleys hosting microbial communities surviving at -60 °C (Cary *et al.*, 2010), the liquid brine veins between sea ice crystals harboring metabolically-active microorganisms at -20 °C (Deming, 2002) or permafrost cryopegs, *i.e.* salty water pockets that have remained liquid at -10 °C for about 100 000 years (Gilichinsky *et al.*, 2005). Psychrophiles and their biomolecules also possess an interesting biotechnological potential, which has already found several applications (Margesin & Feller, 2010).

Cold exerts severe physicochemical constraints on living organisms including increased water viscosity, decreased molecular diffusion rates, reduced biochemical reaction rates, perturbation of weak interactions driving molecular recognition and interaction, strengthening of hydrogen bonds that, for instance, stabilize inhibitory nucleic acid structures, increased solubility of gases and stability of toxic metabolites as well as reduced fluidity of cellular membranes (D'Amico *et al.*, 2006; Gerday & Glansdorff, 2007; Margesin *et al.*, 2008; Rodrigues & Tiedje, 2008). Previous biochemical studies have revealed various adaptations at the molecular level such as the synthesis of cold-active enzymes by psychrophiles or the incorporation of membrane lipids promoting homeoviscosity in cold conditions. It was shown that the high level of specific activity at low temperatures of cold-adapted enzymes is a key adaptation to compensate for the exponential decrease in

chemical reaction rates as the temperature is reduced. Such high biocatalytic activity arises from the disappearance of various non-covalent stabilizing interactions, resulting in an improved flexibility of the enzyme conformation (Feller & Gerday, 2003; Siddiqui & Cavicchioli, 2006; Feller, 2010). Whereas membrane structures are rigidified in cold conditions, an adequate fluidity is required to preserve the integrity of their physiological functions. This homeoviscosity is achieved by steric hindrances introduced into the lipid bilayer via incorporation of *cis*-unsaturated and branched-chain lipids, a decrease in average chain length, and an increase both in methyl branching and in the ratio of *anteiso*- to *iso*-branching (Russell, 2007).

More recently, several genomes from psychrophilic bacteria have been sequenced (Danchin, 2007; Casanueva *et al.*, 2010) but only a few of them have been analyzed with respect to cold adaptation (Saunders *et al.*, 2003; Rabus *et al.*, 2004; Medigue *et al.*, 2005; Methe *et al.*, 2005; Riley *et al.*, 2008; Rodrigues *et al.*, 2008; Allen *et al.*, 2009; Ayala-del-Rio *et al.*, 2010). However, the lack of common features shared by all these psychrophilic genomes has suggested that cold adaptation superimposes on pre-existing cellular organization and, accordingly, that the adaptive strategies may differ between the various microorganisms (Bowman, 2008; Piette *et al.*, 2010).

The Gram-negative bacterium *Pseudoalteromonas haloplanktis* is a typical representative of γ -proteobacteria found in cold marine environments and, in fact, strain TAC125 has been isolated from sea water sampled along the Antarctic ice-shell (Terre Adélie). Such strains thrive permanently in sea water at about -2 °C to +4 °C but are also anticipated to endure long term frozen conditions when entrapped in the winter ice pack. The genome of *P. haloplanktis* TAC125 has been fully sequenced and has undergone expert annotation (Medigue *et al.*, 2005). This work has allowed a proteomic study of its cold-acclimation proteins (CAPs)¹, *i.e.* proteins that are continuously overexpressed at a high level during growth at low temperatures (Piette *et al.*, 2010). This has demonstrated that protein synthesis and protein folding are the main up-regulated functions, suggesting that both cellular processes are limiting factors for bacterial development in cold environments. Furthermore, a proteomic survey of cold-repressed proteins at 4 °C has revealed a strong repression of most heat shock proteins (Piette *et al.*, 2011). This chapter describes the various proteomic features analyzed in the context of adaptation to life at low temperature.

2. Temperature dependence of growth

The ability of *P. haloplanktis* to grow at low temperatures is illustrated in Fig. 1. This psychrophilic Antarctic bacterium maintains a doubling time of ~4 h at 4 °C in a marine broth, with an extrapolated generation time of 5 h 15 at 0 °C (Fig. 1a). This can be compared with the behavior of a mesophilic bacterium such as *E. coli*, which displays a doubling time of ~8h at 15 °C and which fails to grow below ~8 °C (Strocchi *et al.*, 2006). When the culture temperature is raised up to 20 °C, the generation time moderately decreases (*e.g.* 1 h 40 at 18 °C) with a concomitant increase in the biomass produced at the stationary phase (Fig. 1b). At temperatures higher than 20 °C, the doubling time of *P. haloplanktis* slightly increases again with, however, a drastic reduction in cell density at the stationary phase (Fig. 1b), indicating a heat-induced stress on the cell. *P. haloplanktis* TAC125 fails to grow above 29 °C, thereby

¹ The abbreviations used are: CAPs, cold acclimation proteins; CRPs, cold repressed proteins; TF, trigger factor; ROS, reactive oxygen species

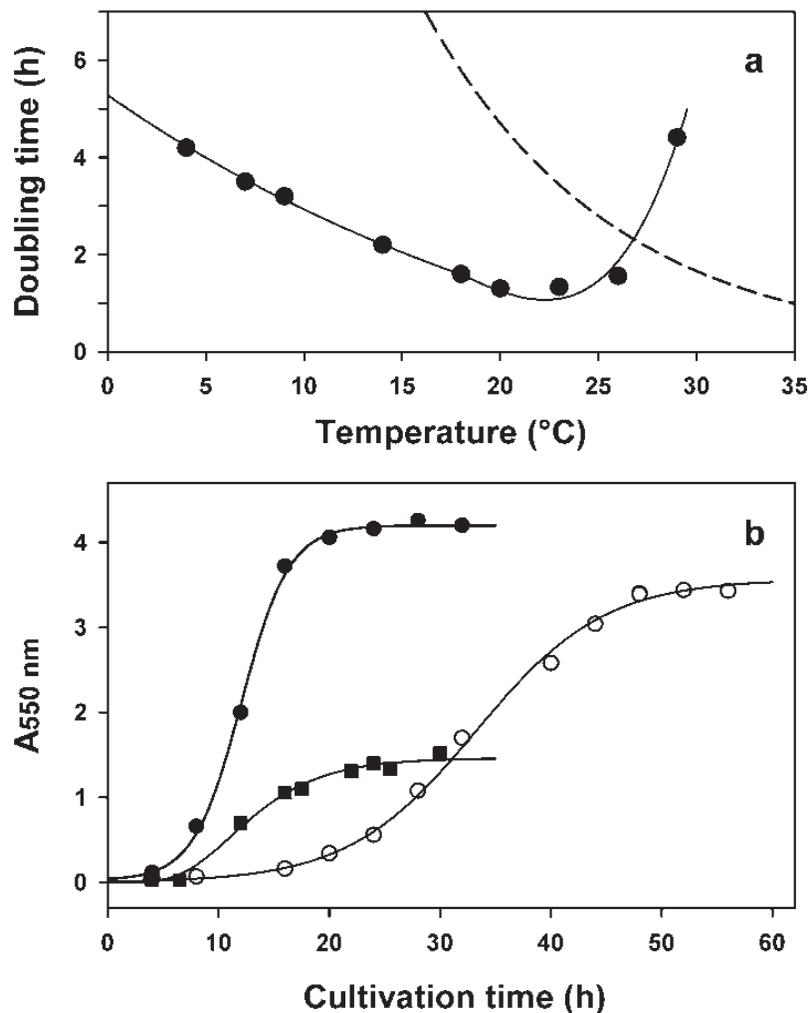


Fig. 1. (a) Temperature dependence of the generation time of *Pseudoalteromonas haloplanktis* TAC125 grown in a marine broth (solid line and circles). A typical curve for *E. coli* RR1 in LB broth is shown for comparison (dashed) (b) Growth curves of *P. haloplanktis* at 4°C (○), 18°C (●) and 26°C (■). Reprinted with permission from Piette *et al.*, 2011. © 2011 American Society for Microbiology.

defining its upper cardinal temperature. According to this growth behavior, the temperatures of 4 °C and 18 °C were selected for the differential comparison of the proteomes, as 18 °C does not induce an excessive stress as far as growth rate and biomass are concerned.

The fast growth rate of the Antarctic bacterium is primarily achieved by a low temperature dependence of the generation times when compared with a mesophilic bacterium, *i.e.* the generation time of *P. haloplanktis* is moderately increased when the culture temperature is decreased (Fig. 1a). It should be stressed that enzymes from cold-adapted organisms are characterized by both a high specific activity at low temperatures and a low temperature dependence of their activity (formally, a weak activation enthalpy), *i.e.* reaction rates of psychrophilic enzymes are less reduced by a decrease in temperature as compared with mesophilic enzymes (D'Amico *et al.*, 2003; Feller & Gerday, 2003). Accordingly, the growth characteristics of the Antarctic bacterium (Fig. 1a) appear to be governed by the properties of its enzymatic machinery: high enzyme-catalyzed reaction rates maintain metabolic fluxes

and cellular functions at low temperatures, whereas the weak temperature dependence of enzyme activity counteracts the effect of cold temperatures on biochemical reaction rates.

3. Cold-induced versus cold-repressed proteins

The proteomes expressed by the Antarctic bacterium at 4 °C and 18 °C during the logarithmic phase of growth have been compared by two-dimensional differential in-gel electrophoresis (2D-DIGE), enabling the co-migration in equal amounts of cell extracts obtained from both conditions (labeled by distinct CyDye fluorophores) in triplicate gels (Fig. 2).

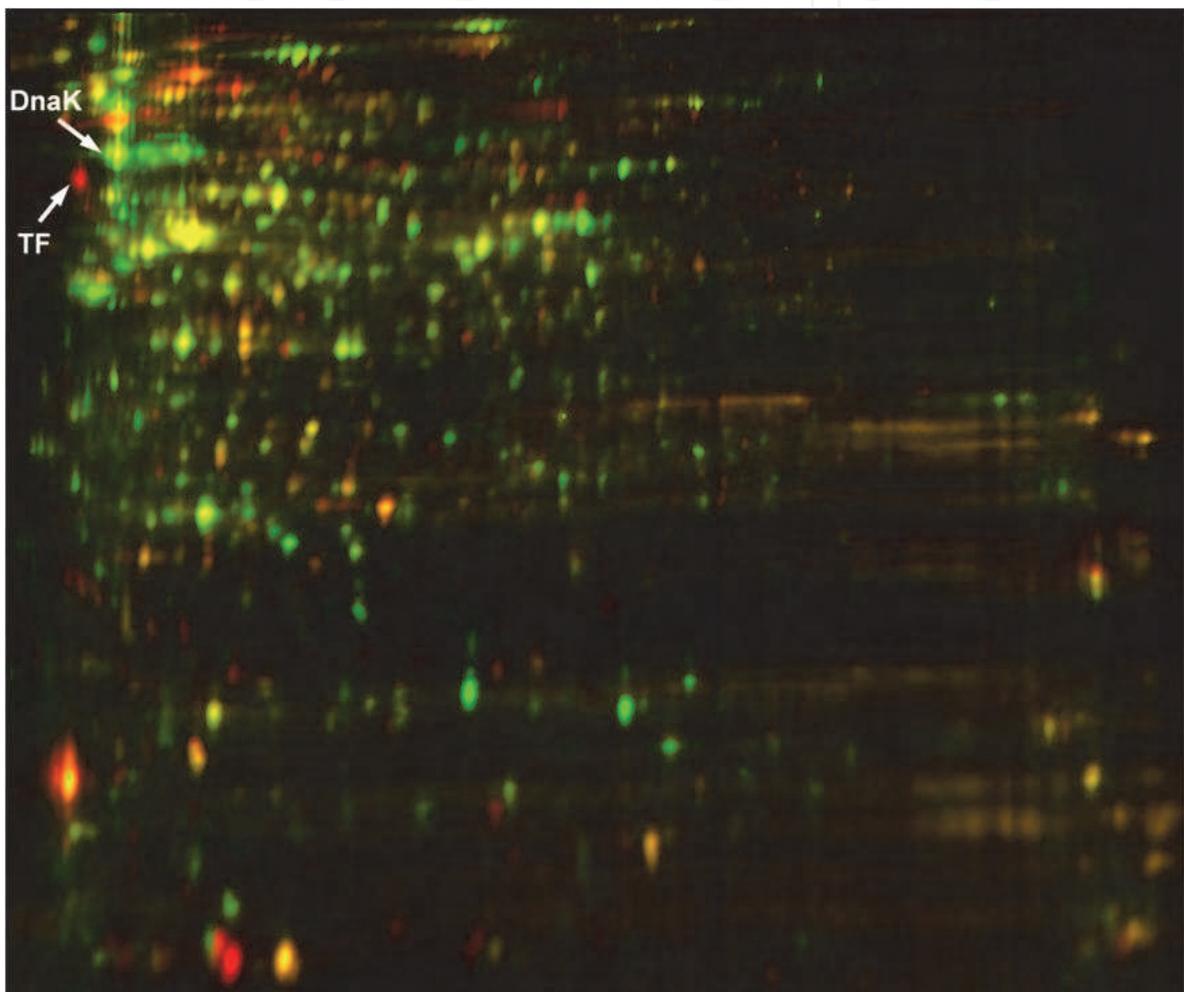


Fig. 2. Comparison of intracellular soluble proteins from *P. haloplanktis* grown at 4°C (red-labeled) and 18°C (green-labeled) on 2D-DIGE gels analyzed by fluorescence. From left to right, non-linear gradient from pH 3 to pH 10. From top to bottom, mass scale from ~150 to ~15 kDa. The intense red fluorescence of the trigger factor (TF) spot correlates with its up-regulation at 4°C, whereas the intense green fluorescence of the DnaK spot correlates with its down-regulation. Adapted with permission from Piette *et al.*, 2010. © 2010 Wiley.

In a typical single 2D-gel (Fig. 3), 142 protein spots are more abundant at 4 °C. As protein extracts were prepared from cells growing exponentially at this temperature, all up-regulated proteins at 4°C are regarded as CAPs. Furthermore, 309 protein spots are less

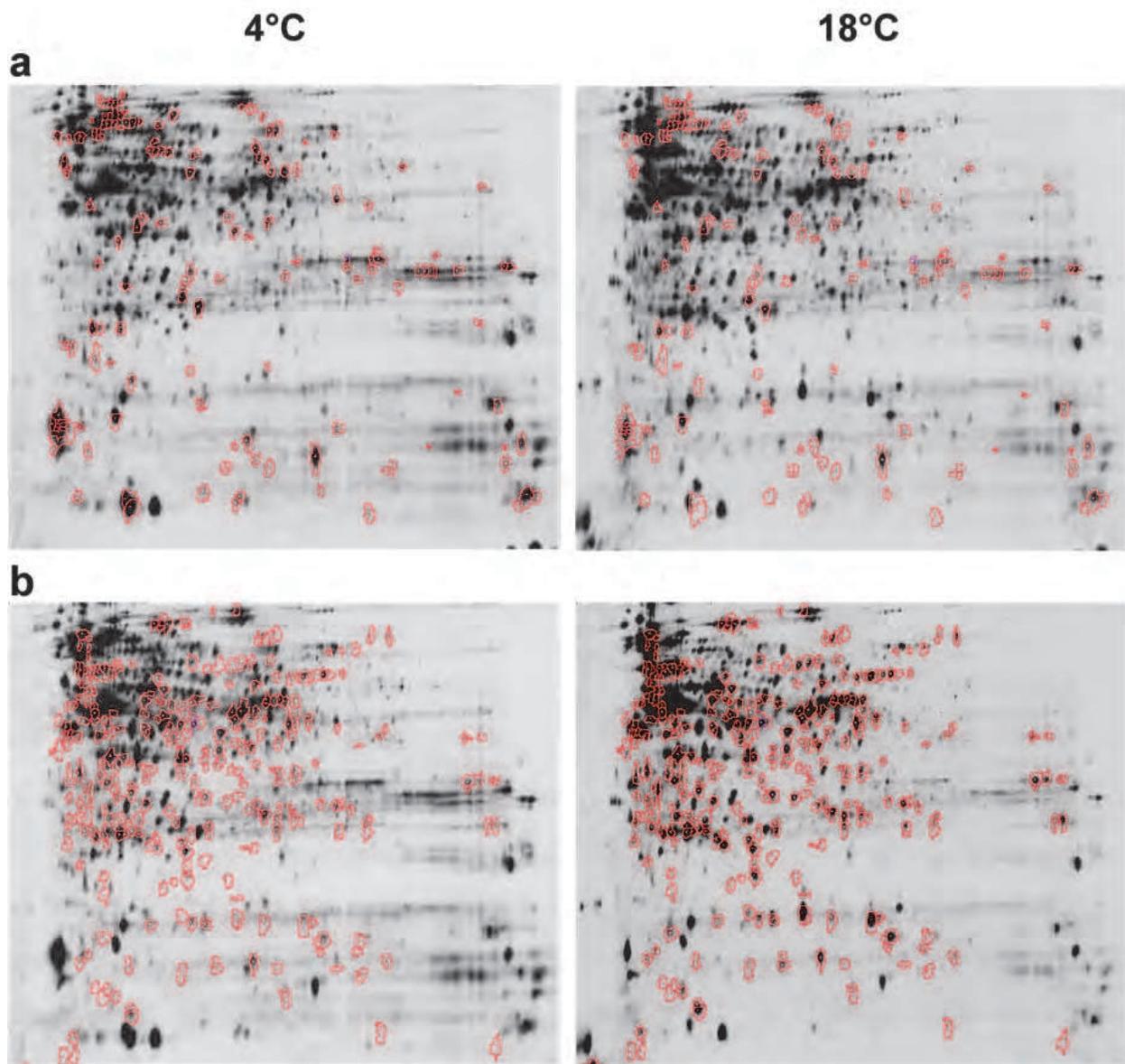


Fig. 3. Differential analyses of soluble cellular proteins from *Pseudoalteromonas haloplanktis* grown at 4°C (left panels) and 18°C (right panels) on 2D-DIGE gels analyzed by fluorescence. **(a)** 142 protein spots that are more intense at 4°C are indicated. **(b)** 309 protein spots that are less intense at 4°C are indicated. Reprinted with permission from Piette *et al.*, 2011. © 2011 American Society for Microbiology.

intense at 4 °C as compared with 18 °C. This unexpected large number of cold-repressed proteins (CRPs) already indicates that numerous cellular functions are down-regulated during growth at low temperature.

The induction factors for CAPs and the repression factors for CRPs, given by the spot volume ratio between 4 °C and 18 °C are illustrated in Fig. 4. This distribution shows that most CAPs and CRPs have a five-time higher or lower relative abundance at 4 °C. However, about 20% of these differentially expressed proteins display up- or down-regulation factors higher than 5, revealing that some key cellular functions are strongly regulated. Amongst all these differentially expressed proteins, 40 CAPs and 83 CRPs were retained, which satisfied both statistical biological variation analysis and mass spectrometry identification scores, as

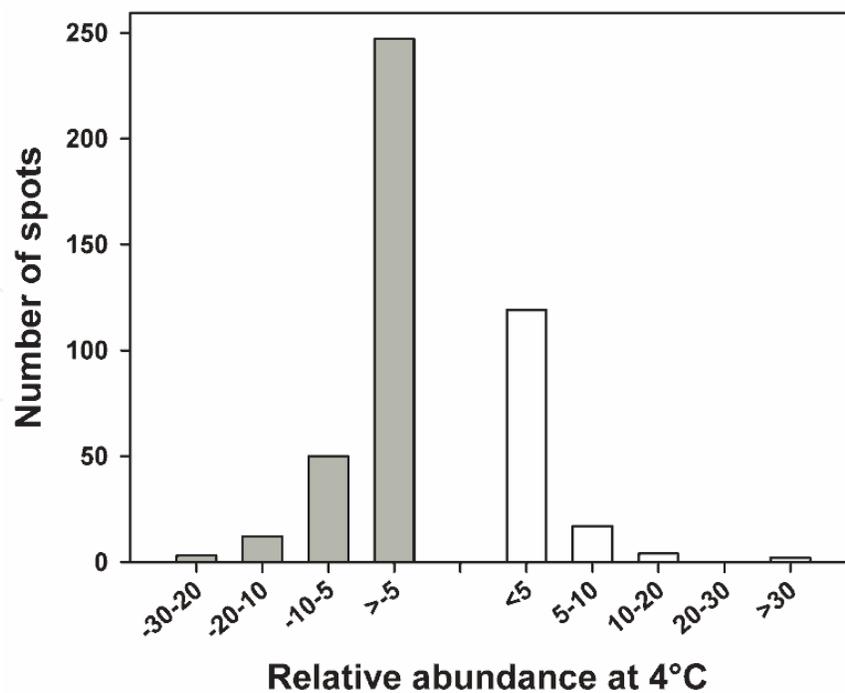


Fig. 4. Distribution of the relative abundance of cold-repressed proteins (dashed, negative values) and of cold acclimation proteins (positive values) in the proteome of *P. haloplanktis* grown at 4°C and 18°C. Reprinted with permission from Piette *et al.*, 2011. © 2011 American Society for Microbiology.

detailed in the original publications (Piette *et al.*, 2010; Piette *et al.*, 2011). Accordingly, the identified proteins should be analyzed as markers of a pathway or of a general function, rather than for their specific function as they represent 27% of the differentially expressed proteins at 4°C.

4. Cold shock and heat shock proteins

One of the most remarkable features of the differentially expressed proteome of *P. haloplanktis* is the strong up-regulation at 4°C of proteins that are regarded as cold shock proteins in mesophilic bacteria, as well as the down-regulation to nearly undetectable levels of proteins classified as heat shock proteins (Fig. 5).

Cold shock proteins that have been identified as CAPs in *P. haloplanktis* include Pnp (+4x), TypA (+5x) and the trigger factor TF (+38x) that are involved in distinct functions (degradosome, membrane integrity and protein folding, respectively). Sustained synthesis of various cold shock protein-homologues has been also reported in other cold-adapted bacteria (Bakermans *et al.*, 2007; Kawamoto *et al.*, 2007; Bergholz *et al.*, 2009). There are therefore striking similarities between the cold shock response in mesophiles and cold adaptation in psychrophiles. From an evolutionary point of view, it can be proposed that one of the adaptive mechanisms to growth in the cold was to regulate the cold shock response, shifting from a transient expression of cold shock proteins to a continuous synthesis of at least some of them. Interestingly, nearly all proteins displaying the highest repression factors at 4°C are heat shock proteins (Rosen & Ron, 2002) including the main chaperones DnaK (-13x) and GroEL (-3.4x), the accessory chaperones such as Hsp90 (-28x), the small heat shock proteins IbpA (-24x) and IbpB (-18x), as well as LysS (-17x).

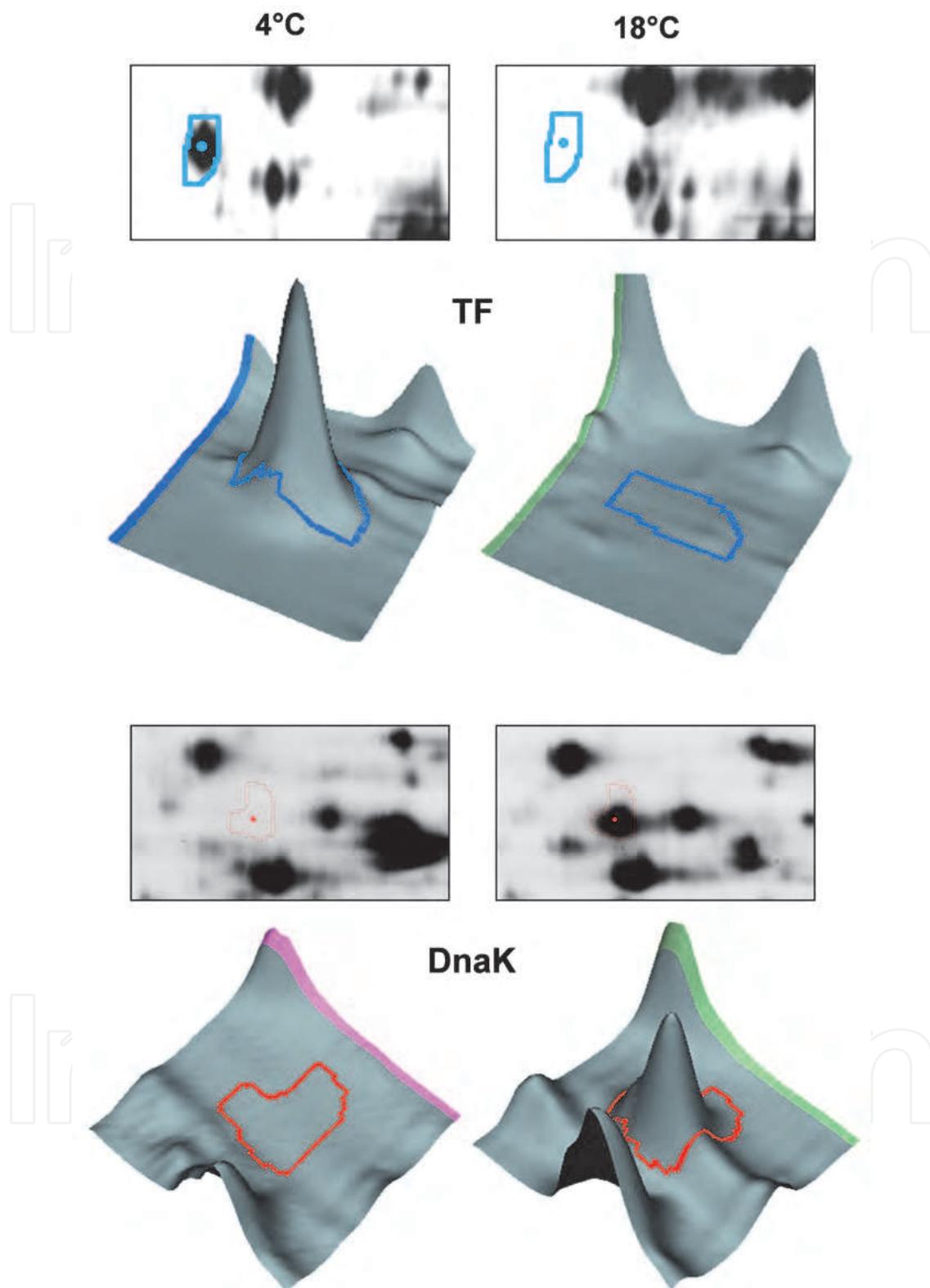


Fig. 5. Comparative analysis of spots containing the trigger factor TF (a cold shock protein) and DnaK (a heat shock protein) from *P. haloplanktis* grown at 4°C (left panels) and 18°C (right panels). Spot views on 2D-gels (circled) and three-dimensional images. Adapted with permission from Piette *et al.*, 2010; Piette *et al.*, 2011. © 2010 Wiley and © 2011 American Society for Microbiology.

In mesophilic bacteria such as *E. coli*, cold shock and heat shock proteins are transiently expressed in response to temperature downshift and upshift, respectively. By contrast, the Antarctic bacterium continuously over-expresses some cold shock proteins (Piette *et al.*, 2010) whereas most heat shock proteins are continuously repressed at 4 °C. It is obvious that regulation of the expression of these proteins involved in thermal stress is a primary adaptation to bacterial growth at low temperatures that remains to be properly explained.

5. Protein folding at low temperature rescued by the trigger factor

In bacteria, the three main chaperones are the trigger factor TF, a cold shock protein that stabilize nascent polypeptides on ribosomes and initiate ATP-independent folding, DnaK that mediates co- or post-transcriptional folding and the GroEL/ES chaperonin that acts downstream in folding assistance (Hartl & Hayer-Hartl, 2009). Both latter chaperones are also well-known heat shock proteins. The trigger factor TF (+38x up-regulated at 4°C) is the first molecular chaperone interacting with virtually all newly synthesized polypeptides on the ribosome. It delays premature chain compaction and maintains the elongating polypeptide in a non-aggregated state until sufficient structural information for productive folding is available and subsequently promotes protein folding (Merz *et al.*, 2008; Hartl & Hayer-Hartl, 2009; Martinez-Hackert & Hendrickson, 2009). Furthermore, TF also contains a domain catalyzing the *cis-trans* isomerization of peptide bonds involving a proline residue (Kramer *et al.*, 2004). This *cis-trans* isomerization is a well-known rate-limiting step in protein folding (Baldwin, 2008). On the other hand the major heat shock proteins were identified as strongly cold-repressed proteins in the proteome of *P. haloplanktis* (or, in other words, they are up-regulated at 18°C). The overexpression of bacterial heat shock proteins at elevated temperatures is well recognized as being indicative of a heat-induced cellular stress (Rosen & Ron, 2002; Goodchild *et al.*, 2005). Although this is obviously relevant for the Antarctic bacterium grown at 18 °C, the implications for the psychrophilic strain appear to be more complex. Indeed, these heat shock proteins are chaperones assisting co- or post-translational protein folding (Hartl & Hayer-Hartl, 2009). Furthermore, it has been demonstrated that GroEL from *P. haloplanktis* is not cold-adapted, it is inefficient at low temperatures as its activity is reduced to the same extent than that of its *E. coli* homologue (Tosco *et al.*, 2003). Accordingly, under this imbalanced synthesis of folding assistants, protein folding at low temperature is apparently compromised in the Antarctic bacterium.

Considering the down-regulation of heat shock chaperones and the inefficiency of GroEL from *P. haloplanktis* at low temperature, as well as the essential function of TF in the initiation of proper protein folding, it can be proposed that TF rescues the chaperone function at low temperatures, therefore explaining its unusual overexpression level. It follows that TF becomes the primary chaperone of the Antarctic bacterium for growth in the cold. Although the psychrophilic bacterium maintains a minimal set of chaperones, this is obviously sufficient to allow bacterial development at low temperature.

6. Possible origins of heat shock protein repression at low temperature

The strong overexpression of TF at low temperature can be understood according to its above mentioned essential function. By contrast, the reasons for the concomitant repression of heat shock chaperones in the Antarctic bacterium remain hypothetical. At least four possible origins, not mutually exclusive, can be mentioned. *i)* Low temperature slows down

the folding reaction and is well known to reduce the probability of misfolding and aggregation (King *et al.*, 1996), therefore possibly reducing the need for heat shock chaperones that act downstream from TF. *ii*) In *E. coli*, it has been shown that synthesis of heat shock proteins is repressed during growth at low temperatures, but also that these heat shock proteins are harmful to cells at 4 °C, as their induced expression reduces cell viability at this temperature (Kandror & Goldberg, 1997). Accordingly, the observed cold-repression of heat shock proteins would be beneficial to the psychrophilic bacterium. *iii*) To our knowledge, the reasons for this harmful effect of heat shock proteins have not been investigated. A possible explanation could be found in the second function of these chaperones. Indeed, besides their role in folding assistance, many heat shock proteins bind partly folded polypeptides and promote their fast degradation by proteases Lon and Clp. In addition, TF enhances the binding affinity of some heat shock proteins for these partly folded polypeptides (Kandror *et al.*, 1995; Kandror *et al.*, 1997). In the case of the Antarctic bacterium, heat shock chaperones would then have an increased ability to bind slowly folding polypeptides at low temperatures and to promote their unwanted degradation: this would account for the cold repression of heat shock chaperones by *P. haloplanktis*. *iv*) The observation that GroEL from the Antarctic bacterium is non cold-adapted (Tosco *et al.*, 2003) suggests that this chaperonin is well suited to function during sudden temperature increases of the environment. Indeed, microorganisms subjected to seasonal or local temperature variations (*e.g.* melting sea ice, polar surface soils, etc.) would advantageously maintain a heat shock response involving non cold-adapted chaperones remaining active at transiently high temperatures.

7. Structural properties of the psychrophilic trigger factor

According to its essential function in *P. haloplanktis*, the psychrophilic TF has been analyzed into more details. Its amino acid sequence (47,534 Da) displays 61% identity (85% similarity) on 434 residues with its homologue from *E. coli*. Its sequence is also close to that of some known TF from psychrophilic bacteria. The pronounced sequence similarity and predicted secondary structure conservation with *E. coli* TF suggest that the psychrophilic chaperone should also fold into an extended “crouching dragon” conformation (Ferbitz *et al.*, 2004) comprising three domains (Fig. 6). The N-terminal domain mediates ribosome attachment *via* an exposed loop, the PPIase activity domain located at the opposite end of the molecule (Kramer *et al.*, 2004) and the C-terminal domain forming the body of the protein and bearing the central module of chaperone activity (Merz *et al.*, 2006).

In order to analyze the psychrophilic TF, its gene has been cloned and overexpressed in *E. coli* and the recombinant protein has been purified to homogeneity (Piette *et al.*, 2010). Its thermal stability was investigated by differential scanning calorimetry. Fig. 7 shows that TF from the Antarctic bacterium is a marginally stable protein, exhibiting a melting point T_m at 33°C. It follows that at a typical mesophilic temperature of 37°C, almost all the protein population is already in the unfolded state. In addition, the calorimetric enthalpy is also very weak ($\Delta H_{cal} = 82.5 \text{ kcal mol}^{-1}$, the sum of all enthalpic contributions to protein stability disrupted during unfolding and calculated from the area under the transition). By comparison, a T_m of 54°C and a calorimetric enthalpy of 178 kcal mol⁻¹ have been reported for the *E. coli* trigger factor analyzed by DSC (Fan *et al.*, 2008). Despite its modular structure, *P. haloplanktis* TF unfolds according to a perfect 2-state transition (Fig. 7), *i.e.* without significantly populated intermediates between the native and the unfolded states. This

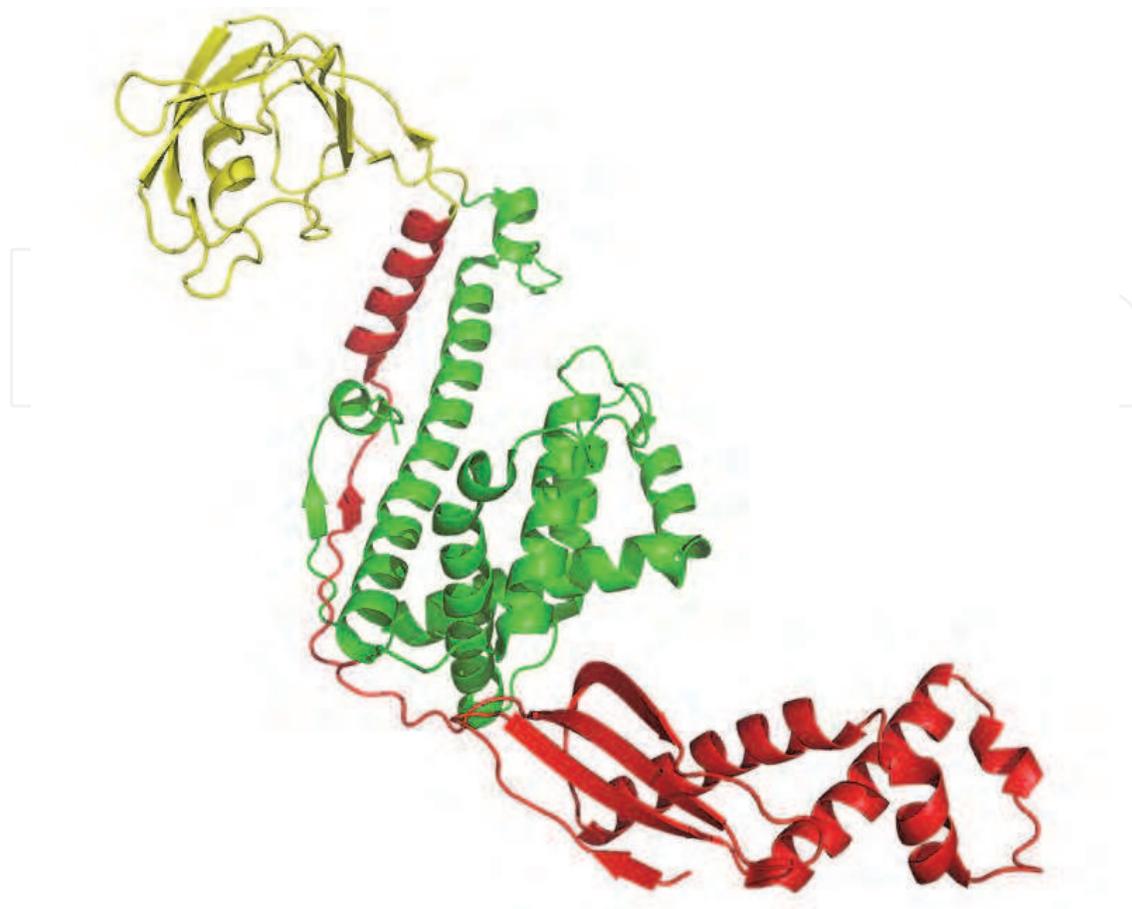


Fig. 6. Domain organization in the trigger factor structure (based on *E. coli* trigger factor: PDB 1W26). The N-terminal domain mediating ribosome attachment (aa 1-144) is in red, the PPIase domain (aa145-247) is in yellow and the C-terminal domain bearing the central module of the chaperone activity (aa 248-432) is in green. As a result of the strong conservation of primary and predicted secondary structures in *P. haloplanktis* TF, a model of its structure built by homology modeling is undistinguishable from the *E. coli* crystal structure. Reprinted with permission from Piette *et al.*, 2010. © 2010 Wiley.

indicates that the psychrophilic TF is uniformly unstable and unfolds cooperatively. To the best of our knowledge, *P. haloplanktis* TF is the least stable protein reported so far. This strongly suggests that the essential chaperone function requires considerable flexibility and dynamics to compensate for the reduction of molecular motions at freezing temperatures.

The *E. coli* trigger factor has been reported to undergo *in vitro* a concentration-dependent dynamic equilibrium between the monomeric and the dimeric forms. Static light scattering experiments performed in batch mode provided a mean particle mass of 51 kDa and 106 kDa for *P. haloplanktis* and *E. coli* TF, respectively. This is in agreement with a monomeric psychrophilic TF and a dimeric *E. coli* TF. However, in dynamic light scattering the particle polydispersity (size distribution) of *P. haloplanktis* TF was twice that of *E. coli* TF, suggesting that the psychrophilic TF may possibly perform transient intermolecular interactions. These observations are in line with a report showing that the trigger factor from the psychrophile *Psychrobacter frigidicola* is a monomeric chaperone (Robin *et al.*, 2009). The interpretation of these differences in oligomerization state remains to be properly explained but suggest noticeable differences between psychrophilic and mesophilic bacteria for the TF function in

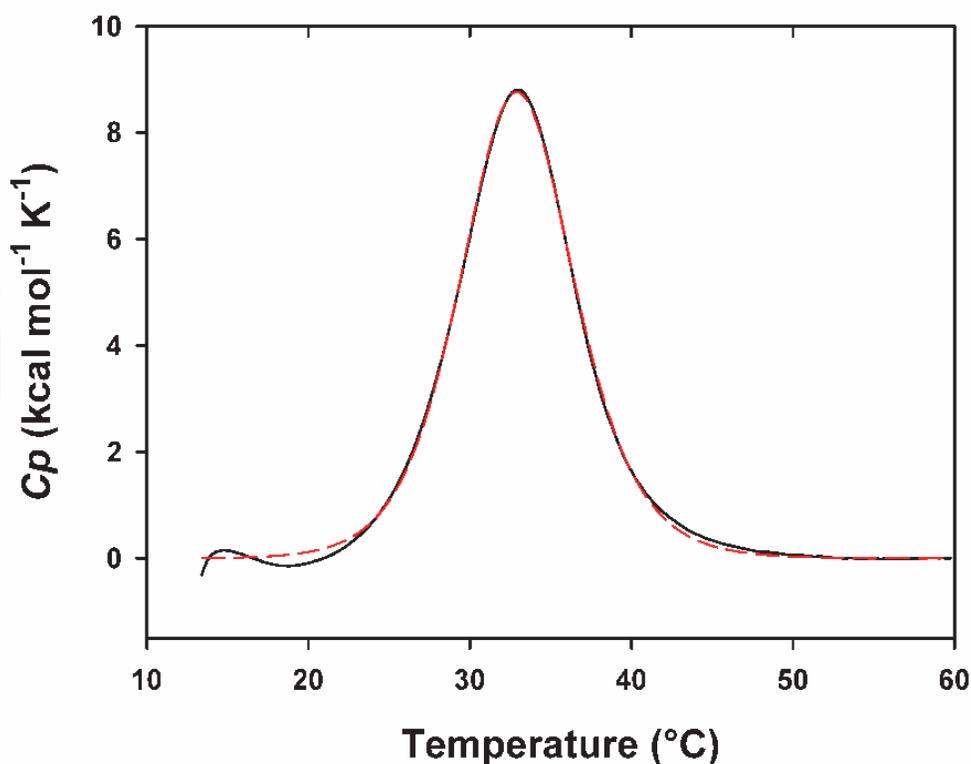


Fig. 7. Microcalorimetric analysis of the trigger factor from *P. haloplanktis*. The melting point T_m corresponds to the top of the transition at 33°C. The calorimetric enthalpy ΔH_{cal} corresponds to the area under the transition. The red dashed line corresponds to the fit of the DSC data to a two-state unfolding transition. Baseline-subtracted data have been normalized for protein concentration (2.6 mg/ml in 30 mM Mops, 250 mM NaCl, pH 7.6). Adapted with permission from Piette *et al.*, 2010. © 2010 Wiley.

the cytoplasmic fraction, when not bound to the ribosome. Finally, in a typical refolding assay monitoring chaperone activity, it has been found that *P. haloplanktis* TF is inactive at 20°C and recovers partial activity at 15°C. It was also shown that this TF requires near-zero temperatures (Fig. 8) to efficiently bind an unfolded protein (Piette *et al.*, 2010). This illustrates a remarkable cold adaptation of the chaperone function in the psychrophilic TF.

8. Protein synthesis and folding are limiting factors in the cold

Thirty percent of the identified CAPs are directly related to protein synthesis and cover all essential steps, from transcription (including RNA polymerase RpoB) to translation and folding (TF, PpiD). Amongst these CAPs, for instance, genes *pnp* and *rpsA* encode components of the degradosome that regulates transcript lifetimes. The Rho termination factor is a RNA/DNA helicase that can contribute to relieve nucleic acid secondary structures strengthened in cold conditions. Interestingly, mutations in the ribosomal protein L6 (RplF) have been reported to cause loss of *E. coli* cells viability at 0°C (Bosl & Bock, 1981) and it is also a CAP in *P. haloplanktis*. Methionyl-tRNA synthetase MetG displays one of the highest up-regulation ratio (+7.6x): this can be tentatively related to the requirement of an increased pool of initiation tRNA to promote protein synthesis. Two putative proteases were also identified as CAPs and can potentially participate to proteolysis of misfolded proteins.

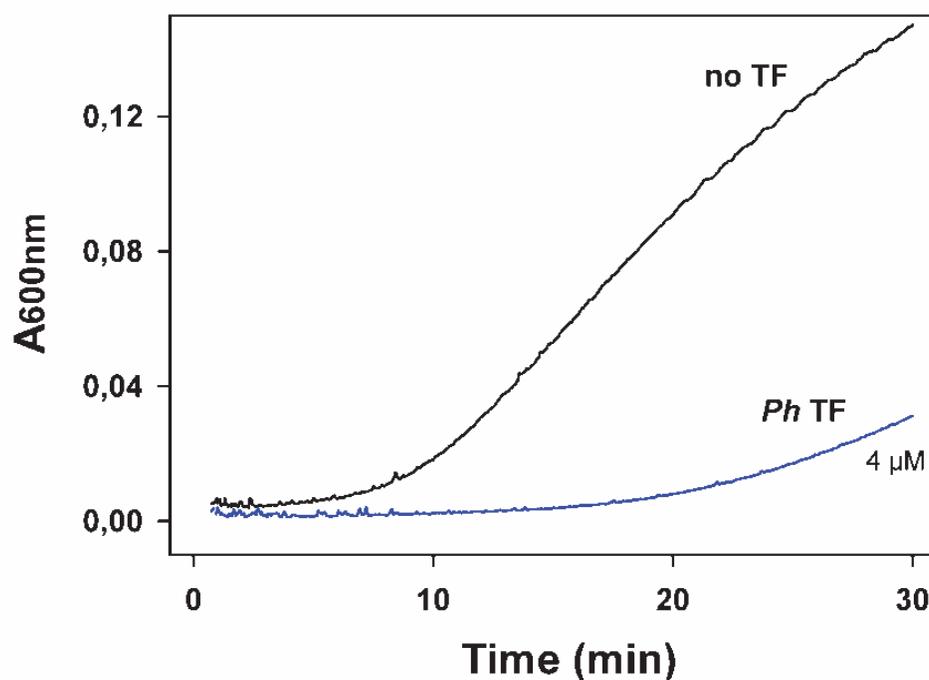


Fig. 8. Detection of the chaperone activity of the psychrophilic TF. Aggregation at 15°C of a chemically unfolded protein (glyceraldehyde-3-phosphate dehydrogenase) is monitored by absorbance at 600 nm after incubation in melting ice. In the absence of TF (black trace), aggregation occurs as soon as the temperature is raised. By contrast, TF suppresses aggregation for 10-15 min (blue trace). Adapted with permission from Piette *et al.*, 2010. © 2010 Wiley

In the last step of protein synthesis, the folding catalyst TF acts on proteins synthesized by the ribosome and also catalyses peptidyl-prolyl *cis-trans* isomerisation (PPIase) while PpiD (another PPIase) is involved in the folding of outer membrane proteins. Peptidyl-prolyl *cis-trans* isomerisation appears therefore as a limiting factor for a wide range of proteins in *P. haloplanktis*. Furthermore, some previous studies on cold-adapted microorganisms have reported either PPIases (Goodchild *et al.*, 2004b; Suzuki *et al.*, 2004) or the trigger factor (Qiu *et al.*, 2006; Kawamoto *et al.*, 2007) as potential CAPs. It seems therefore that the constraints imposed by protein folding in the cold are common traits in several psychrophilic microorganisms.

Altogether, these observations strongly suggest that low temperatures impair protein synthesis and folding, resulting in up-regulation at 4°C of the associated cellular processes.

9. Metabolism depression at low temperatures

Nearly half of down-regulated proteins at 4°C are related to superclasses of function involved in the bacterial general metabolism. This includes the degradation or biosynthesis of compounds and the production of energy. Most of these proteins belong to the oxidative metabolism, in particular to glycolysis, the pentose phosphate pathway, Krebs cycle and electron chain transporters. Accordingly, the Antarctic bacterium depresses its general metabolism when grown at low temperature. This is in agreement with the reduced biomass produced at 4 °C as compared with cultures run at 18 °C (Fig. 1b). As mentioned in the previous section, protein synthesis and folding are limiting factors for the growth of *P.*

haloplanktis at cold temperatures (Piette *et al.*, 2010). However, the proteomic data indicates that when these limitations are alleviated at 18 °C, the bacterium proliferates by activation of its general metabolism and therefore divides actively and produces more biomass. The high number of identified ribosomal proteins and of elongation factors involved in translation indicates that protein synthesis is no longer limiting but is also stimulated at 18 °C.

10. Down-regulation of iron metabolism at low temperatures

Iron uptake and iron-related proteins are clearly down-regulated at 4 °C in *P. haloplanktis*. The uptake of this essential element in an aquatic environment is mediated by several iron transport systems. Two systems were found to be down-regulated at 4 °C: the ABC transporter (FbpA) and a TonB-dependent receptor. The first is involved in the uptake of the weakly soluble ferric ion (Fe^{3+}) directly from the environment and the second is required for the transport of heme complexes and ferric siderophores through the membrane (Clarke *et al.*, 2001). The reduced needs for iron by *P. haloplanktis* at 4 °C can be partly explained by the down-regulation of the Krebs cycle and respiratory chain (and their iron-containing complexes such as SdhB), by the repression of HmgA, which requires Fe^{2+} to degrade cyclic amino-acids or by the strong down-regulation of catalase (which is made up of four heme groups). Hemes are tetrapyrroles that have porphobilinogen as a precursor: this is in agreement with the down-regulation of both GltX (glutamyl-tRNA synthetase) and HemB (5-aminolevulinate dehydratase), which are responsible for porphobilinogen synthesis.

Various metallic ions are essential to the cell metabolism and therefore the fact that proteomic data only points to cold repression of iron-related proteins is puzzling. Iron in a redox-active form (Fe^{2+}) is potentially deleterious, as it is able to induce oxidative cell damage by the Fenton reaction, for instance (Valko *et al.*, 2005). It can be tentatively proposed that, as a result of the improved stability of ROS (reactive oxygen species) at low temperatures, the down-regulation of iron-related proteins could contribute to an avoidance of such detrimental iron-based reactions. In this respect, it should be mentioned that the genome of *P. haloplanktis* entirely lacks the ubiquitous ROS-producing molybdopterin metabolism (Medigue *et al.*, 2005). This suggests that the Antarctic bacterium tends to avoid ROS production involving metallic ions.

11. Oxidative stress-related proteins

The pattern of oxidative stress-related proteins in *P. haloplanktis* is complex because some have been identified as CAPs, while others were found to be CRPs. For instance, glutathione synthetase is the second main up-regulated protein at 4°C (+13.2x) and superoxide dismutase (+1.6x) was also detected as a CAP. This is a clear indication of a cellular response to an oxidative stress arising from increased dioxygen solubility and ROS stability. On the other hand, the second group of proteins that displays the highest repression factors at 4 °C is represented by the oxidative stress-related proteins catalase (-6.5x), glutathione reductase (-8.1x) and peroxiredoxin (-15.7x). At first sight, this may be regarded as a conflicting result because conclusive evidences have indicated that psychrophiles are exposed to a permanent oxidative stress at low temperatures, which originates from improved dioxygen solubility and increased ROS stability (Rabus *et al.*, 2004; Medigue *et al.*, 2005; Methe *et al.*, 2005; Duchaud *et al.*, 2007; Bakermans *et al.*, 2007; Ayub *et al.*, 2009; Piette *et al.*, 2010). In order to reconcile these apparent contradictions, it should be recalled that the general aerobic

metabolism of the Antarctic bacterium is stimulated at 18 °C, also resulting in ROS production. Accordingly, the identified oxidative stress-related proteins would be better regarded as being induced at 18 °C, rather than repressed at 4 °C.

The up-regulation of catalase and peroxiredoxin at 18°C shows that the bacterium needs to be protected against ROS like H₂O₂ as both enzymes catalyze its decomposition into O₂ and H₂O. Under oxidative stress, the NADPH supply for reduced glutathione regeneration is also dependent on glucose-6-phosphate dehydrogenase (Zwf) in the first step of the pentose phosphate pathway, and indeed Zwf is positively regulated at 18 °C. Glutathione reductase (Gor) plays a central role in the reoxidation of NADPH from the pentose phosphate pathway, allowing formation of reduced glutathione, an important cellular antioxidant. The up-regulated DNA-binding DPS protein (DpsB) plays a major role in the protection of bacterial DNA from damage by ROS and is induced under stress conditions. Some DPS proteins are also able to bind iron and are involved in its storage and in the protection of the cell (Haikarainen & Papageorgiou, 2010).

There is obviously a finely-tuned balance between the cellular mechanisms protecting against oxidative stresses generated by low temperatures (resulting from ROS stability and oxygen solubility) and by high temperatures (resulting from stimulated metabolic activity). The number of identified proteins does not allow a detailed description of this balance but the strong involvement of glutathione synthetase, glutathione reductase and the

Microorganism	Source	Technique	Ref.
<i>Bacillus psychrosaccharolyticus</i>	Soil, marshes	2D-PAGE	1
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	LC/LC-MS/MS	2
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	2D-PAGE	3
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	ICAT LC MS	4
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	LC MS	5
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	SDS-PAGE LC-MS/MS	6
<i>Exiguobacterium sibiricum</i>	Siberian permafrost	2D LC MS	7
<i>Shewanella livingstonensis</i>	Antarctic seawater	2D-PAGE	8
<i>Psychrobacter cryohalolentis</i>	Siberian permafrost	2D-PAGE	9
<i>Psychrobacter articus</i>	Siberian permafrost	2-D HPLC & MS	10
<i>Moritella viscosa</i>	Atlantic salmon	2D-PAGE	11
<i>Psychrobacter articus</i>	Siberian permafrost	2D-PAGE	12
<i>Sphingopyxis alaskensis</i>	Alaska seawater	GeLC-MS/MS	13
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	LC/LC-MS/MS	14
<i>Lactococcus piscium strain</i>	seafood products	2D-PAGE	15
<i>Pseudoalteromonas haloplanktis</i>	Antarctic seawater	2D-DIGE	16
<i>Methanococcoides burtonii</i>	Ace Lake, Antarctica	8-plex iTRAQ	17
<i>Acidithiobacillus ferrooxidans</i>	Mine drainage, Canada	2D-PAGE	18

Table 1. Proteomic studies performed on psychrophilic microorganisms. References: 1, Seo *et al.*, 2004; 2, Goodchild *et al.*, 2004a; 3, Goodchild *et al.*, 2004b; 4, Goodchild *et al.*, 2005; 5, Saunders *et al.*, 2005; 6, Saunders *et al.*, 2006; 7, Qiu *et al.*, 2006; 8, Kawamoto *et al.*, 2007; 9, Bakermans *et al.*, 2007; 10, Zheng *et al.*, 2007; 11, Tunsjo *et al.*, 2007; 12, Bergholz *et al.*, 2009; 13, Ting *et al.*, 2010; 14, Williams *et al.*, 2010; 15, Garnier *et al.*, 2010; 16, Piette *et al.*, 2010; Piette *et al.*, 2011; 17, Williams *et al.*, 2011; 18, Mykytczuk *et al.*, 2011.

identification of enzymes belonging to the pentose phosphate pathway suggest that regulation of the cytoplasmic redox buffering capacity via glutathione is a key component.

12. Other proteomic studies

A selection of recent proteomic studies on psychrophilic and cold-adapted microorganisms is listed in Table 1. It is worth mentioning that the CAPs and CRPs identified in these studies do not constitute a conserved set of proteins in terms of identification and expression level. Nevertheless, a survey of these data shows that the main upregulated functions for growth at low temperatures are protein synthesis (transcription, translation), RNA and protein folding, membrane integrity and transport, antioxidant activities and regulation of specific metabolic pathways. Such heterogeneous upregulation of CAPs supports the view that cold-adaptation mechanisms are constrained by the species-specific cellular structure and organization, resulting in distinct adaptive strategies. This hypothesis is based on a previous observation made by Bowman (2008). In a review of genome data from psychrophiles, he concluded that the lack of common features shared by these genomes suggests that cold adaptation superimposes on pre-existing cellular organization and, accordingly, that the adaptive strategies may differ between the various microorganisms.

13. Conclusions

The capacity of psychrophilic bacteria to thrive successfully in permanently cold environments obviously requires a vast array of adaptations. At least two prerequisites to this environmental adaptation can be cited: *i*) from a functional standpoint, the synthesis of cold-active enzymes is required to support the bacterial metabolism and its energy production (Feller & Gerday, 2003; Siddiqui & Cavicchioli, 2006; Feller, 2010), and *ii*) from a structural standpoint, the synthesis of cold-adapted lipids is required to maintain the cell membrane integrity, fluidity and functions (Russell, 2007). It should be noted that the first adaptation is genetically encoded in the protein sequence (and results from a long term adaptation), whereas the second adaptation involves regulation of pre-existing biosynthetic pathways. However, neither of these basic adaptations is sufficient because low temperature induces physicochemical constraints that are unavoidable but that can be attenuated by cellular mechanisms. For instance, low temperature reduces molecular diffusion rates and also increases water and cytoplasmic viscosity. It can be proposed that both physicochemical constraints are responsible for the rate limiting steps of *P. haloplanktis* growth in the cold, namely protein synthesis and folding, as deduced from proteomic experiments. Indeed, bacterial protein synthesis is one of the most complex cellular processes and requires diffusion and docking of numerous partners with ribosomes (mRNA, tRNA, initiation factors, elongation factors, GTP...). Assuming a high, cold-active ribosomal efficiency (although this has not been demonstrated to date), its synthetic activity would be nevertheless restricted by diffusion and availability of the required partners. The rate of protein folding is also limited by low temperatures. This is an entropically-driven process governed by the chemical nature of the polypeptide chain and of water molecules. Furthermore, the main protein chaperones are not catalysts *per se* but rather they assist in protein folding and prevent or relieve misfolding. The above mentioned physicochemical constraints exert their effects on all psychrophiles and it can be anticipated that protein

synthesis and folding are also limiting for these microorganisms, unless specific adaptive mechanisms have been developed.

Considering the constraints on protein folding, one would expect the activation of the full set of protein chaperones. By contrast, we found that the Antarctic bacterium strongly over-expresses the trigger factor (a cold-shock protein in *E. coli*) and represses the major chaperones (also HSPs in *E. coli*) at 4 °C. Interestingly, the same trend has been reported for *E. coli* grown at low temperatures (Kandror & Goldberg, 1997). This antagonism between cold-shock and heat-shock chaperones appears to be a common feature in these bacteria, but the origin of this antagonism remains hypothetical as discussed in section 6.

Increased dioxygen solubility and ROS stability is another physicochemical constraint exerted on psychrophilic microorganisms. Indeed, we have noted the activation of oxidative stress protection mechanisms in *P. haloplanktis* grown at 4 °C. Furthermore, the repression of iron-related proteins at 4 °C seems to be related to the avoidance of Fenton-type reactions (Valko *et al.*, 2005). The genome of *P. haloplanktis* also reveals several insights into ROS protection such as deletion of ROS producing pathways, several occurrences of dioxygenases and the repair mechanisms of oxidized compounds (Medigue *et al.*, 2005). Similar observations have been made in the genome and proteome of other psychrophilic microorganisms (Rabus *et al.*, 2004; Medigue *et al.*, 2005; Methe *et al.*, 2005; Duchaud *et al.*, 2007; Bakermans *et al.*, 2007; Ayub *et al.*, 2009; Piette *et al.*, 2010), revealing a general constraint on these bacteria. However, we found that at 18 °C, another type of oxidative stress is induced by stimulation of metabolic activity. This balance between cold-induced and heat-induced oxidative stresses deserves further investigation.

Our proteomic data points to a global reduction of the general metabolism in the Antarctic bacterium at 4 °C. If the behavior of the psychrophilic bacterium were extrapolated at near freezing temperatures, it is anticipated that its metabolism would be further depressed. It is worth mentioning that ancient bacteria survival has been reported in frozen samples of up to half a million years old and such viability has been correlated with the capacity to slowly repair DNA (Johnson *et al.*, 2007). The temperature dependence of the metabolic pattern in psychrophilic bacteria can thus be summarized as follows. During the prevailing cold conditions in polar environments, these bacteria remain metabolically active (Deming, 2002) but their growth can be limited by some temperature-sensitive cellular processes (protein synthesis and folding in the case of *P. haloplanktis*). When the environmental temperature transiently increases, both their metabolism and cell division are stimulated. Besides an elemental thermodynamic effect on the cell unit, this can also be regarded as an adaptive strategy to increase the viable population during short warmer periods. By contrast, at extremely low temperatures or during long-term freezing survival, these bacteria evolve towards a dormancy state with minimal cellular metabolic activity aimed at preserving the cell's genetic program (Johnson *et al.*, 2007). Various exogenic protective mechanisms have also been proposed such as secreted exopolymers (Krembs & Deming, 2008) or particulate matter association (Junge *et al.*, 2004). From an ecological point of view, and in the context of a possible global warming, a rise in the environmental temperature would mainly result in the proliferation of bacteria such as *P. haloplanktis*.

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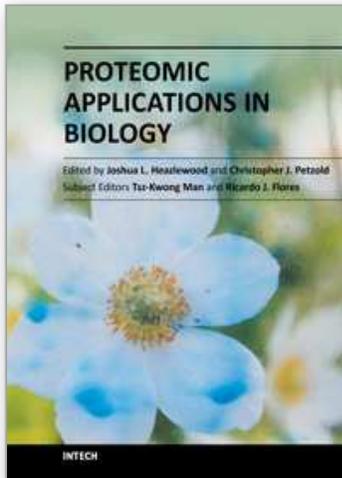
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The past decade has seen the field of proteomics expand from a highly technical endeavor to a widely utilized technique. The objective of this book is to highlight the ways in which proteomics is currently being employed to address issues in the biological sciences. Although there have been significant advances in techniques involving the utilization of proteomics in biology, fundamental approaches involving basic sample visualization and protein identification still represent the principle techniques used by the vast majority of researchers to solve problems in biology. The work presented in this book extends from overviews of proteomics in specific biological subject areas to novel studies that have employed a proteomics-based approach. Collectively they demonstrate the power of established and developing proteomic techniques to characterize complex biological systems.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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